

The prebiotic effect of *Anoectochilus formosanus* and its consequences on bone health

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Abstract

The present study evaluated the prebiotic effect of a standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and its effects on osteoporosis in ovariectomised (OVX) rats. The OVX rats were randomly divided into five groups and orally treated with water, SAEAF (200 and 400 mg/kg daily) and inulin (400 mg/kg daily) for 12 weeks. The sham group was orally treated with water. The SAEAF treatment enhanced the number of faecal bifidobacteria in OVX rats. The results of a Ca-balance experiment showed that SAEAF increased apparent Ca absorption and retention. The OVX rats were killed after SAEAF treatment lasting 12 weeks. The SAEAF decreased the caecal pH values and increased the caecal wall weight, caecal mucosa calbindin-D9k mRNA expression, free-Ca concentration and levels of SCFA in the caecum. The mineral content, density and biomechanical strength of bones were lower in OVX rats than the sham group, but these bone losses were prevented by SAEAF administration. Microtomography scanning showed that the SAEAF-treated rats had higher trabecular bone volume than the OVX rats. These results suggest that SAEAF prevented bone loss associated with ovarian hormone deficiency in the rats.

Key words: *Anoectochilus formosanus*: Prebiotics: Calcium balance studies: Osteoporosis

Prebiotics are non-digestible food ingredients that benefit the host by selectively stimulating the favourable growth and/or activity of one or more indigenous probiotic bacteria that could deliver potential beneficial health effects⁽¹⁾.

Microbial fermentation products of prebiotics, such as SCFA, are responsible for an increase in Ca absorption in the large intestine. A high concentration of SCFA in the caecum leads to decreased caecal pH, which increases the concentration of soluble Ca⁽²⁾. In addition, butyrate, a SCFA, belongs to a new class of anti-osteoporotic agents that may be useful in treating bone loss^(3–5). Furthermore, several reports have indicated that the ingestion of prebiotics such as inulin and fructo-oligosaccharides might help prevent osteoporosis^(2,6).

Anoectochilus formosanus (Orchidaceae) is an orchidaceous perennial herb, of which the entire plant has been used as a folk medicine for treating underdevelopment in children in Taiwan. This traditional use of the herb suggests that it might enhance Ca absorption⁽⁷⁾. Several studies in rats have found that *A. formosanus* ameliorated the osteoporosis induced by ovariectomy (OVX)^(8,9). Masuda *et al.*⁽⁹⁾ have shown that ethanolic extracts of *A. formosanus* suppress the

bone loss otherwise caused by oestrogen deficiency, by inhibiting osteoclast formation. Our previous study has shown that aqueous extracts of *A. formosanus* ameliorated bone loss caused by OVX by stimulating bone formation⁽⁸⁾.

A bioactivity-guided fractional study of the use of *A. formosanus* in mice has shown that the ethyl acetate fraction enhances hepatitis induced by carbon tetrachloride⁽¹⁰⁾. The present study developed a dietary supplement from *A. formosanus* to prevent bone loss. A standardised aqueous extract of *A. formosanus* (SAEAF) was prepared, excluding the ethyl acetate fraction as described⁽¹¹⁾. In Taiwan, because SAEAF is used as a raw material in functional food, its safety has been evaluated.

The use of *A. formosanus* in supplementary treatment (e.g. treating stunted growth in children) has led to speculation that the herb might enhance Ca absorption, with a possible relation to prebiotic action. The SAEAF was found to be a potential prebiotic in a pilot study that evaluated the effects of SAEAF on intestinal bacteria. Once there, SAEAF was used for *in vitro* fermentation for strains, such as bifidobacteria, lactobacilli, *Escherichia coli* and clostridia. The results

Abbreviations: γ -GT, γ -glutamyl transpeptidase; CaBP, calbindin-D9K; CFU, colony-forming units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IPAF, indigestible polysaccharide; OCN, osteocalcin; OVX, ovariectomy; SAEAF, standardised aqueous extract of *Anoectochilus formosanus*.

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indicated that SAEAF could selectively enhance the population of bifidobacteria in *in vitro* fermentation.

The prebiotic action may be caused by polysaccharides from SAEAF. The present study, therefore, investigated whether prebiotic activity was involved in the anti-osteoporotic effects of SAEAF in OVX rats. Previous research on prebiotics has most often investigated inulin, a β -(1 \rightarrow 2) fructan⁽¹⁾; thus, we used it as a positive control in the present study.

Materials and methods

Preparation of a standardised aqueous extract of *Anoectochilus formosanus*

The *A. formosanus* plants were purchased from Yu-Jung Farm. The plants were identified by the Institute of Chinese Pharmaceutical Sciences, China Medical University (Taiwan), where a plant specimen (no. CMCP 1253) has been deposited.

Fresh, whole plants of cultured *A. formosanus* were extracted with distilled water, and the filtrate was partitioned with ethyl acetate. The aqueous fraction was further filtered and evaporated under reduced pressure to yield a purpuric residue, termed SAEAF. The SAEAF yield was approximately 2.8%.

Isolation and characteristic of polysaccharides

A 4-fold volume of 95% ethanol was added to the solution of SAEAF for precipitating the polysaccharides from SAEAF. The precipitate was collected by centrifugation. The precipitate was treated by total dietary fibre assay kit (Megazyme) to remove the protein and starch content to obtain indigestible polysaccharide (IPAF). The IPAF was determined for protein and carbohydrate contents by modified Bradford and phenol-sulphuric acid methods^(12,13). It was stored in ethanol at 4°C until further use. In addition, the molecular structure of IPAF was roughly determined by Fourier transform IR spectrometer (Shimadzu IR 21, Shimadzu Corporation)⁽¹⁴⁾.

Animals

Wistar female rats (3 months old) were purchased from BioLASCO Company, Limited. The study protocols complied well with the institutional guidelines of the China Medical University for the use of laboratory animals. The animals were housed in an air-conditioned room (21–24°C) under 12 h of light (07.00–19.00 hours) and were allowed free access to food pellets and water throughout the study.

Surgical procedure

Female rats were anaesthetised with sodium pentobarbital (40 mg/kg, intraperitoneally; Siegfried), and their ovaries were removed bilaterally. The rats in the sham-operated group were anaesthetised, laparotomised and sutured without removing their ovaries. The OVX rats were allowed to lose bone for 4 weeks. At 4 weeks post-OVX, the OVX rats were randomly divided into five groups (*n* 8) and orally administered water, SAEAF (200 and 400 mg/kg daily) or

inulin (Sigma Aldrich; 400 mg/kg daily) for 12 weeks. The sham-operated group was orally treated with water. The body weight of each animal was measured once a week until the final day of administration.

Studies on the prebiotic activity of standardised aqueous extract of *Anoectochilus formosanus* in ovariectomised rats

To determine bacteria in faeces, fresh faeces were collected directly from each rat on the 28th day after SAEAF administration, weighed and poured into a dilute solution of peptone saline with cysteine (0.5 g/l; Wako) immediately⁽¹⁵⁾. After homogenising the faeces, serial decimal dilutions were prepared, avoiding aeration. Bifidobacteria iodoacetate medium-25 agar was used for enumeration of *Bifidobacterium* spp.⁽¹⁶⁾ and tryptose-sulfite-D-cycloserine agar (Oxoid) was used for quantification of *Clostridium perfringens*⁽¹⁷⁾. Bifidobacteria iodoacetate medium-25 and tryptose-sulfite-D-cycloserine agar were cultured anaerobically in an atmosphere of 5% CO₂ and 7% H₂ in N₂ at 37°C for 72 h. The results were expressed as log colony-forming units (CFU)/g faeces.

Studies on calcium balance in ovariectomised rats after administration of standardised aqueous extract of *Anoectochilus formosanus*

The method used for studying Ca balance was based on our previous work⁽¹⁸⁾. During the period spanning days 37 and 42 after SAEAF administration, all the rats were housed in individual metabolism cages containing a grid-floor and a facility for separate collection of faeces and urine. To acclimatise the animals to the new environment, the rats were placed in these cages 2 d before the beginning of a 4 d metabolic study aimed at the determination of net dietary Ca absorption.

Food consumption was then monitored on a daily basis over the 4 d period of the metabolic balance study. Urine and faecal samples (24 h) of each animal were collected, and the volume of urine for each animal was recorded. Portions of the urine samples were acidified with 12 M-HCl and stored at –20°C until analysis. The faecal samples of each animal were dried overnight at 100°C. The diets and dried faecal samples were ash-dried at 700°C for 12 h. The feed and faecal ashes were solubilised with 6 M-HCl (Scharlau) for the Ca assay. Total Ca was measured by the *o*-cresolphthalein complexone method using a commercial kit (Randox). Apparent Ca absorption and apparent Ca balance were calculated using equations 1 and 2, respectively⁽¹⁸⁾.

$$\text{Apparent Ca absorption} = \text{Ca intake} - \text{faecal Ca}, \quad (1)$$

$$\text{apparent Ca balance} = \text{Ca intake} - \text{faecal Ca} - \text{urinary Ca}. \quad (2)$$

Sampling procedure

On the last day of the study (day 84 from SAEAF administration), the animals were killed under deep anaesthesia with a high dose of sodium pentobarbital (65 mg/kg, intraperitoneally). After sampling of blood, the caeca (with their

contents), vaginae, femurs and tibiae were also removed. The vaginae were taken from the vaginal opening to cervix and weighed immediately. The pH of the caecum was measured immediately using a pH meter (Model IQ 150, Spectrum Technologies). After pH measurement, the caeca were stored instantly at -80°C until Ca and SCFA analysis. Blood was centrifuged at 2000 *g* at 4°C for 15 min to separate the plasma. The dissected bones were stored at -80°C until examination.

The caecal contents were transferred into a sterile tube and defrosted on ice. The caecal walls were flushed clean with ice-cold saline, blotted on filter paper and weighed to give the caecal wall weight. The caecal contents were shaken in a vortex mixer for 30 s and then centrifuged at 4°C at 9000 *g* for 10 min. The supernatants of the caecal contents were divided into two parts. One part was used for the free-Ca measurement using commercial kits (Randox), while the other part was diluted with 0.0085 M-sulphuric acid (Showa) and filtered using a 0.22 μm filter (Millipore) for SCFA analysis using HPLC. The HPLC system consisted of a 709 IC pump (Metrohm), Rheodyne sample injector (Rheodyne) and Super CO-150 column heater (Enshine). The peaks were detected by a Shodex RI-71 detector (Showa Denko) using a Transgenomic ICsep ION-300 column (300 \times 7.8 mm, Transgenomic) at 65°C and eluted with 0.0085 M-sulphuric acid at a flow rate of 0.4 ml/min. The standards used for SCFA determination were lactate, acetic acid, propionic acid and butyric acid that were obtained from Sigma Aldrich.

RT-PCR analysis

The mucosal cells of the caecum were scraped. Total RNA was isolated from the mucosal cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method, as described by Chomczynski & Sacchi⁽¹⁹⁾. A 3 μg sample of total RNA was subjected to RT with moloney murine leukaemia virus RT in a 50 μl reaction volume. Aliquots of the RT mix were used for amplification of fragments of calbindin-D9K (CaBP), a cholecalciferol-induced Ca-binding protein, by PCR. The primers for rat CaBP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were 5'-AAGAGCATTTTCAAAAATA-3' and 5'-GTCTCAGAA-TTGCTTATT-3' (product size, 314 bp), and 5'-TGTGTCCG-TCGTGGATCTGA-3' and 5'-CCTGCTTACCACCTTCTTGA-3' (product size, 76 bp), respectively. The expression levels of all the transcripts were normalised to that of the GAPDH mRNA in the same tissue sample. The PCR products were separated on a 2% agarose gel and recorded on Polaroid film; the bands were quantified with a densitometer.

Biochemistry

Enzyme-linked immunosorbent assay kits, based on rat osteocalcin (OCN) enzyme immunoassay (Biomedical Technologies, Inc.), were used to analyse serum OCN concentrations. Urinary γ -glutamyl transpeptidase (γ -GT) and creatinine were assayed using clinical test kits (Roche Diagnostics) in a spectrophotometric analyser. The γ -GT contents in urine samples were expressed as units per mmol of urinary creatinine.

Biomechanical three-point bending testing

Bone strength was measured on intact left femurs using a three-point bending test⁽²⁰⁾. Each specimen was placed on two supports spaced 25 mm apart, and load was applied to the bone midway between the supports at a deformation rate of 0.05 mm/s until a fracture occurred. The load-deformation curves were recorded during the bending process using a testing machine (RTI-TST, Royalty Technology). Load-displacement data were used for further computations of the extrinsic material properties, including maximal load, energy to maximal load and linear stiffness. Energies to maximal load were computed as the areas under the load-deformation curves. Stiffness was computed as the slope of the linear portion of the load-deformation curve.

The failure sites of all bone specimens were photographed. Cross-sectional parameters were measured from the photographs (Image-Pro Plus version 5.1; Media Cybernetics) and used in the calculation of the elastic modulus (Young's modulus). The elastic modulus was calculated by the method of Turner & Burr⁽²¹⁾.

Measurement of bone mineral content and bone mineral density

The bone mineral content and bone mineral density of each right tibia were measured using a dual-energy X-ray absorptiometer (XR-26; Norland) using a model for small subjects.

Measurement of trabecular bone microarchitecture by microtomography

The femurs were carefully removed of soft tissue and preserved in 75% alcohol until scanning. The trabecular bone microarchitecture of the distal right femoral metaphysis was

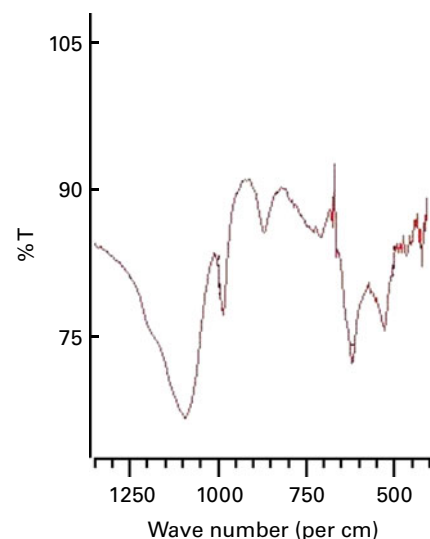


Fig. 1. Fourier-transform IR spectrum of indigestible polysaccharide in potassium bromide pellet scanned between 1350 and 400/cm. %T, percentage transmittance. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

measured using a microtomography scanner (SkyScan 1076, SkyScan), with an isotropic resolution of 18 µm in all three spatial dimensions. For analysing purpose, the region of interest volume of the trabecular bone was selected as 100 slices, thirty slices away from the growth plate of femur to the proximal direction. The region of interest volume was analysed without the cortical bone. The bone volume and the tissue volume were measured directly from the original three-dimensional images, and the trabecular bone volume (bone volume/tissue volume (%)) was normalised to compare samples of different sizes. The other parameters of trabecular structure studied were trabecular number, trabecular thickness and trabecular separation, which were calculated directly from the three-dimensional images.

Statistical analysis

In tables, the results were expressed as means with their pooled standard errors and in figures, they were presented as means and 95% CI. All experimental data were analysed using one-way ANOVA with Dunnett’s test. Values of $P < 0.05$ were considered statistically significant.

Results

Chemical composition

The yield rate of SAEAF to whole plant was approximately 2.8%. The IPAF was 0.25% (w/w) from SAEAF. The IPAF contained 58% carbohydrate and 0.2% protein, indicating that the IPAF was mainly composed of carbohydrates. Fig. 1 shows the Fourier transform-IR profile of the IPAF. The IPAF contained certain special functional groups, indicating that the IPAF was structurally a polysaccharide⁽¹⁴⁾. The peak at 1040/cm was the C–O of the C–O–C stretching vibration. The peak at 892/cm was galactose with β-linkage. We presumed the IPAF as a polysaccharide with a galactan backbone.

Effect of standardised aqueous extract of *Anoectochilus formosanus* on faecal *Bifidobacterium* and *Clostridium perfringens* levels in ovariectomised rats

At 4 weeks after SAEAF administration, no differences were observed in the faecal numbers of *Bifidobacterium* between the OVX + water and sham groups (9.2 (SEM 0.1) log CFU/g faeces). The faecal number of *C. perfringens* also showed no difference between the OVX + water and sham groups (6.2 (SEM 0.1) log CFU/g faeces). The OVX rats that had received SAEAF (400 mg/kg) or inulin for 4 weeks displayed significantly increased faecal numbers of *Bifidobacterium* (9.6 (SEM 0.18) log CFU/g faeces, $P < 0.001$ or 9.6 (SEM 0.1) log CFU/g faeces, $P < 0.001$, respectively). The number of *C. perfringens* in OVX rats that had received SAEAF (400 mg/kg; 6.0 (SEM 0.2) log CFU/g faeces, $P < 0.001$) or inulin (6.1 (SEM 0.1) log CFU/g faeces, $P < 0.01$) was significantly decreased compared with the OVX + water group.

Effects of standardised aqueous extract of *Anoectochilus formosanus* on intestinal absorption and retention of calcium in ovariectomised rats

The experiment on Ca balance was conducted between days 37 and 42 after SAEAF administration. Table 1 shows a summary of the results from the Ca-balance study. Gain in body weight was higher in OVX + water rats than in the sham rats. However, no differences were found between the OVX + water and sham groups for food intake, Ca intake and faecal and urinary Ca contents. Calculation of Ca absorption and retention using the measures of Ca intake and faecal and urinary Ca contents indicated that these processes were reduced in the OVX + water rats relative to the sham group. However, the differences did not attain a level of statistical significance. The OVX rats showed a decrease in Ca absorption and retention of 28.8 and 33.1%, respectively.

No differences were found for body weight, food intake and faecal and urinary Ca contents among the OVX groups

Table 1. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on intestinal absorption and retention of calcium in rats‡
(Mean values with their pooled standard errors, n 8)

Items	Sham	OVX				Pooled SEM
		Water	SAEAF (200 mg/kg)	SAEAF (400 mg/kg)	Inulin (400 mg/kg)	
Body weight (g)	331.4	381.6††	369.4	386.5	375.6	10.8
Food intake (g)	17.6	19.5	18.2	19.9	21.8	0.7
Ca intake (mg)	164.5	182.0	169.6	185.3	203.0	7.1
Faecal Ca (mg)	135.4	161.3	151.2	148.9	164.9	6.8
Urinary Ca (mg)	1.6	2.0	1.5	1.8	3.7	0.4
Absorption (mg)	29.1	20.7	18.4	36.4*	38.1*	4.5
Retention (mg)	27.5	18.4	17.1	34.8*	34.5*	4.2

OVX, ovariectomy.

* Mean values were significantly different compared with the OVX + water group by Dunnett’s test ($P < 0.05$).

†† Mean value was significantly different compared with the sham group ($P < 0.01$).

‡ During the period spanning days 37 and 42 after SAEAF administration, all the rats were housed in individual metabolism cages containing a grid-floor and a facility for separate collection of faeces and urine. To acclimatise the animals to the new environment, the rats were placed in these cages 2 d before the beginning of a 4 d metabolic study aimed at the determination of net dietary Ca absorption.

Table 2. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on caecal wall weight, pH of caecal content and caecal free calcium concentration in ovariectomised (OVX) rats†
(Mean values with their pooled standard errors, *n* 8)

Items	Sham	OVX				Pooled SEM
		Water	SAEAF (200 mg/kg)	SAEAF (400 mg/kg)	Inulin (400 mg/kg)	
Caecal wall weight (g)	1.1	0.96	1.19	1.31**	1.32**	0.05
Caecal pH	6.37	6.53	6.41	6.25**	6.33**	0.04
Free Ca (mg/l)	358	335	358	407***	389**	9

Mean values were significantly different compared with the OVX + water group by Dunnett's test: ** $P < 0.01$, *** $P < 0.001$.

† On the last day of the study, the animals were killed. After sampling of blood, the caeca were also removed. The pH of the caecum was measured immediately using a pH meter. The caecal walls were flushed clean with ice-cold saline, blotted on filter paper and weighed to give the caecal wall weight. The caecal contents were shaken in a vortex mixer for 30 s and then centrifuged. The supernatants of the caecal contents were used for the free-Ca measurement using commercial kits.

(Table 1). However, Ca absorption and retention were higher in groups treated by SAEAF (400 mg/kg) or inulin than in the OVX + water group. The OVX rats treated with SAEAF (400 mg/kg) showed a 76 and 89% increase in Ca absorption and retention, respectively. Similarly, OVX rats treated with inulin showed an increase in Ca absorption and retention (84 and 87%, respectively).

Effects of standardised aqueous extract of Anoectochilus formosanus on body and vaginal weights in ovariectomised rats

At 4 weeks after the operation, the OVX rats showed significant increases in body weight compared with the sham-operated rats ($P < 0.01$). The rats in all four OVX groups exhibited similar body weights before the administration of SAEAF or inulin. At 16 weeks after OVX, the OVX rats continued to show a significantly increased body weight (409.9 (SEM 13.4) g) compared with the sham group (365.6 (SEM 16.4) g) ($P < 0.05$). The increased body weight of the OVX rats was not affected by SAEAF or inulin administration.

The vaginal weights significantly decreased in the OVX rats (136.6 (SEM 1.4) mg) compared with the sham-operated rats (210.9 (SEM 9.8) mg). Decreased vaginal weights in the OVX rats were not affected by SAEAF or inulin administration.

Effects of standardised aqueous extract of Anoectochilus formosanus on caecal wall weight, pH of caecal contents and caecal free-calcium concentration in ovariectomised rats

No significant differences were found in the caecal wall weights, caecal pH and caecal free-Ca concentrations between OVX + water and sham groups (Table 2). Treatment with SAEAF (400 mg/kg) or inulin led to increases in the caecal wall weight of 36.4 and 21.5%, respectively, and to increases in the caecal free-Ca concentrations of 37.5 and 19.4%, respectively. The pH values of the caecal contents were lower in the groups treated with SAEAF (400 mg/kg) or inulin than in the OVX + water group. The caecum content weight, corrected for body weight, was found not to differ significantly between the sham (1.3 (SEM 0.3) g/100 g body weight) and OVX + water groups (1.2 (SEM 0.27) g/100 g body weight). Similarly, no differences were observed between the OVX + water group and the groups treated with OVX + SAEAF or OVX + inulin, respectively.

Effects of standardised aqueous extract of Anoectochilus formosanus on caecal butyrate and total SCFA levels in ovariectomised rats

The results of SCFA analysis showed that butyrate and total SCFA (lactate, acetate, propionate and butyrate) levels in the

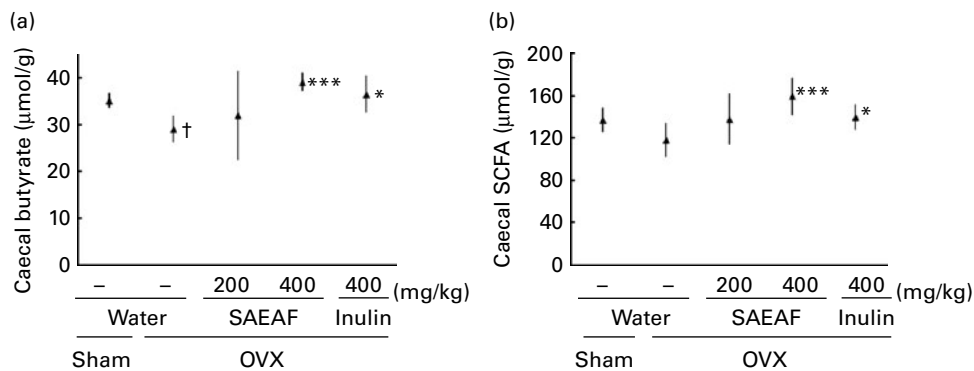


Fig. 2. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on (a) caecal butyrate and (b) total SCFA levels in sham and ovariectomised (OVX) rats. The supernatants of the caecal contents were diluted with sulphuric acid and filtered for SCFA analysis using HPLC. Values are means and 95% CI (*n* 8). Mean values were significantly different compared with the OVX + water group by Dunnett's test: * $P < 0.05$, *** $P < 0.001$. † Mean values were significantly different compared with the sham group ($P < 0.05$).

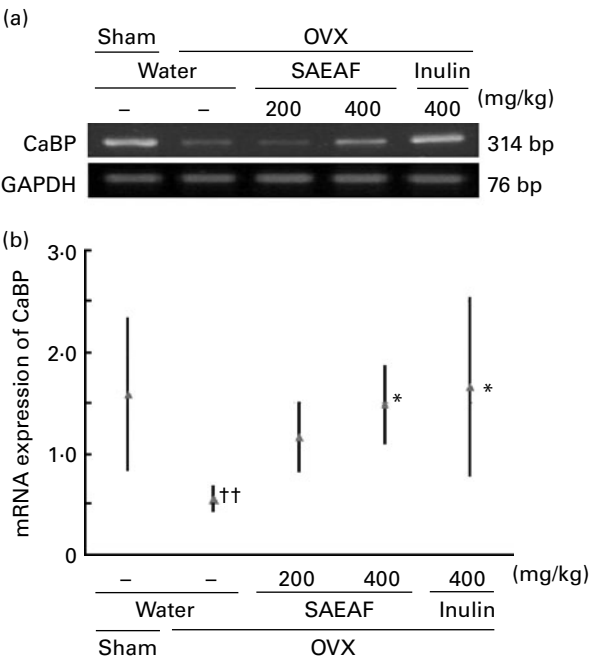


Fig. 3. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on caecal mucosa calbindin-D9K (CaBP) mRNA expression in sham and ovariectomised (OVX) rats. (a) RT-PCR analysis of the expression of CaBP in control rats, and in rats treated with SAEAF (200 or 400 mg/kg) or inulin. Expression of the amplified fragment corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for comparison. The fragments reflect the pooled data for eight samples. (b) Densitometric analyses of CaBP expression after normalisation against GAPDH. Values are means and 95% CI ($n = 8$). * Mean values were significantly different compared with the OVX + water group by Dunnett's test ($P < 0.05$). †† Mean values were significantly different compared with the sham group ($P < 0.01$).

caecal contents were similar in the sham and OVX + water groups (Fig. 2). Caecal levels of total SCFA and butyrate increased by 1.35-fold in each case for the group treated with SAEAF (400 mg/kg), and by 1.18-fold and 1.21-fold, respectively, in the inulin-treated group, compared with the OVX + water group (Fig. 2).

Effect of standardised aqueous extract of Anoectochilus formosanus on expression of caecal mucosa calbindin-D9K mRNA in ovariectomised rats

The fragments shown in Fig. 3 reflect the pooled data for eight samples. Fragments of the *CaBP* gene were amplified by RT-PCR (Fig. 3(a)). The CaBP:GAPDH ratio in the OVX + water group was 68% lower than that in the control group (Fig. 3(b)). Treatment with SAEAF (400 mg/kg) and inulin led to increases of 2.7- and 3.0-fold, respectively, in the CaBP:GAPDH ratio, compared with that of the OVX + water group.

Effects of standardised aqueous extract of Anoectochilus formosanus on plasma osteocalcin and urinary γ -glutamyl transpeptidase levels in ovariectomised rats

The OVX induced an increase in plasma OCN concentration and the urinary γ -GT level in the OVX group compared

with the sham group. Treatment with SAEAF (400 mg/kg) or inulin lowered OCN and γ -GT levels, compared with the OVX + water group (Fig. 4).

Effects of standardised aqueous extract of Anoectochilus formosanus on biomechanical parameters of the femur in ovariectomised rats

The femoral three-point bending test indicated that maximal load, stiffness, energy to maximal load and the Young's modulus (elastic modulus) of the femur were significantly lower in the OVX + water group compared with the sham group (Table 3). Treatment of OVX rats with SAEAF (400 mg/kg) or inulin prevented this post-surgical decline for all four variables (Table 3).

Effects of standardised aqueous extract of Anoectochilus formosanus on tibial bone mineral content and bone mineral density in ovariectomised rats

The bone mineral content and bone mineral density of the tibia were, respectively, 7.3 and 28.5% lower in the OVX + water group than in the sham group. Treatment with SAEAF or inulin significantly prevented a reduction in both the bone mineral content and bone mineral density levels (Fig. 5).

Effects of standardised aqueous extract of Anoectochilus formosanus on bone microarchitecture in distal femoral metaphysis in ovariectomised rats

As expected, OVX rats displayed a lower trabecular bone volume, trabecular thickness and trabecular number, and higher trabecular separation in the distal femur compared with the sham group (Fig. 6 and Table 4). Treatment of OVX rats with SAEAF (400 mg/kg) or inulin prevented the decreases in trabecular bone volume, number of trabeculae and thickness of the trabeculae that were otherwise induced by OVX. Trabecular separation was also reduced by the administration of SAEAF and inulin.

Discussion

The present study demonstrated the bifidogenic activity of SAEAF. Thus, SAEAF displayed a prebiotic character. The IPAF such as inulin are usually regarded as prebiotic materials⁽¹⁾. To guarantee the reproducibility of the present experiment on bioactivity, we isolated and characterised the polysaccharides with a galactan backbone from SAEAF. In addition, SAEAF effectively inhibited OVX-induced osteoporosis in rats. Prebiotic activity is involved in the anti-osteoporotic mechanisms of SAEAF.

Gut bacteria are composed of several hundred different species that include both beneficial and potentially deleterious bacteria, in a delicate balance. Bifidobacteria are recognised as beneficial bacteria, whereas clostridium is regarded as a harmful bacterium⁽²²⁾. Supplementation with probiotics entails the uptake of potentially beneficial micro-organisms that colonise the intestines to improve both the composition and function

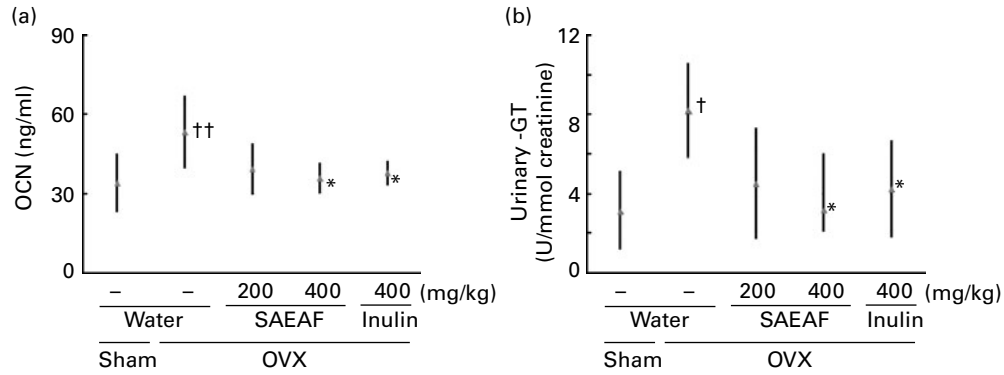


Fig. 4. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on (a) plasma osteocalcin (OCN) and (b) urinary γ -glutamyl transpeptidase (γ -GT) levels in sham and ovariectomised (OVX) rats. Plasma OCN concentration was assessed by using enzyme immunoassay kits. The urinary γ -GT and creatinine levels was assessed by using clinical kits. Values are means and 95% CI (n 8). * Mean values were significantly different compared with the OVX + water group by Dunnett's test ($P < 0.05$). Mean values were significantly different compared with the sham group: † $P < 0.05$, †† $P < 0.01$.

of the intestinal flora. Most probiotic products currently include various bifidobacteria species in particular. Efficient prebiotics require a specific fermentation process that alters the microflora composition towards a more beneficial community structure. This process results from stimulating potentially health-promoting genera, but not the harmful groups⁽²³⁾.

Ovariectomised rats mimic middle-aged post-menopausal women because of the oestrogen deficiency that results from OVX. The present study found no difference between OVX rats and the sham group for the faecal number of bifidobacteria and clostridia. However, SAEAF treatment selectively stimulated the growth of beneficial bifidobacteria and decreased the growth of harmful clostridia in OVX rats. This finding suggests that SAEAF contains prebiotic-like components.

Prebiotics are fermentable, indigestible carbohydrates and the end products of carbohydrate fermentation are SCFA⁽²⁴⁾. A high concentration of organic acids in the caecum leads to a decrease in the caecal pH, which in turn increases the concentration of soluble Ca⁽²⁴⁾. Lopez *et al.*⁽²⁴⁾ reported hypertrophy of the caecum in rats fed with prebiotics. The present study found that SAEAF treatment in OVX rats increased the caecal wall weight and the caecal soluble-Ca concentration, in addition to a decrease in the caecal pH. This finding provides further support for the hypothesis that SAEAF possesses a prebiotic character.

Ca is absorbed in the intestine through two pathways: (1) an active transcellular pathway and (2) a passive

paracellular pathway⁽²⁵⁾. Active transport is dominant only at low luminal Ca concentrations, whereas at higher luminal Ca concentrations passive transport becomes dominant⁽²⁴⁾. The intestinal transcellular Ca transport system has been found to comprise several steps; a major factor is CaBP, a cholecalciferol-induced Ca-binding protein⁽²⁶⁾. A direct correlation exists between the mucosal amounts of CaBP and the efficiency of Ca absorption⁽²⁵⁾. Hence, an increase in mucosal CaBP would indicate an increase in Ca absorption from the intestinal segment. The CaBP is present in the duodenum, the proximal jejunum and caecum⁽²⁷⁾. The present study found that SAEAF treatment increased the caecal free-Ca concentration and mucosal expression of CaBP mRNA, indicating that both the active and passive pathways of Ca absorption were stimulated. In addition, hypertrophy of the caecum was induced by SAEAF; thus, the absorptive surface of the caecum was increased.

The present study evaluated the effects of SAEAF as a treatment for post-menopausal osteoporosis, using a rat OVX model. Oestrogen deficiency has been reported to cause an increase in body weight and a decrease in vaginal weight in OVX rats^(28,29). The SAEAF treatment did not reverse the post-menopausal symptoms of obesity and vaginal atrophy, indicating that SAEAF may not exert oestrogen receptor-agonist activity.

Furthermore, Ca balance has been reported to be affected in OVX rats because of an increase in intestinal Ca secretion

Table 3. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on biomechanical parameters of left femur in ovariectomised (OVX) rats‡

(Mean values with their pooled standard errors, n 8)

Items	Sham	OVX				Pooled SEM
		Water	SAEAF (200 mg/kg)	SAEAF (400 mg/kg)	Inulin (400 mg/kg)	
Maximal load (N)	101.0	86.4††	100.6	103.8*	102.3*	1.8
Stiffness (N/mm)	208.4	153.4†††	172.0	204.1**	189.5*	3.6
Energy (MJ)	66.0	57.4††	62.5	64.5*	64.6*	7.1
Young's modulus (GPa)	21.3	15.5†††	16.6	19.1***	19.2***	0.1

Mean values were significantly different compared with the OVX + water group by Dunnett's test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mean values were significantly different compared with the sham group: †† $P < 0.01$, ††† $P < 0.001$.

‡ Bone strength was measured on intact left femurs using a three-point bending test. Each specimen was placed on two supports spaced 25 mm apart, and load was applied to the bone midway between the supports at a deformation rate of 0.05 mm/s until a fracture occurred.

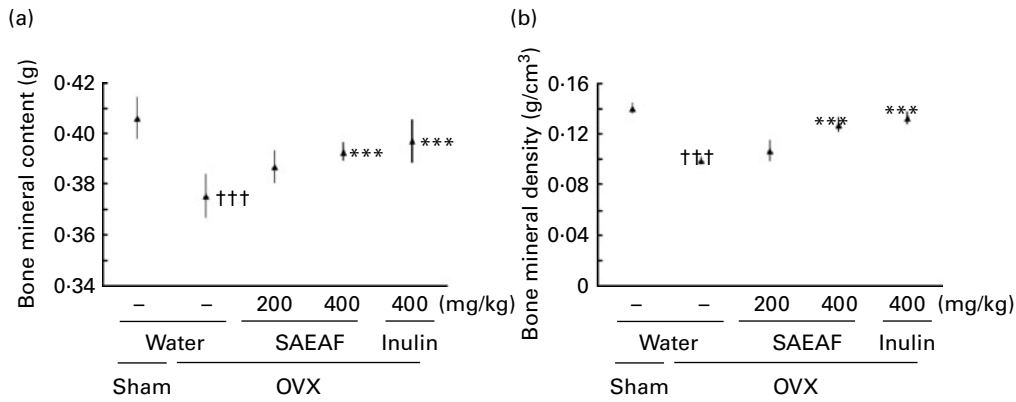


Fig. 5. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on the (a) bone mineral content and (b) bone mineral density of the tibia in sham and ovariectomised (OVX) rats. The bone mineral content and bone mineral density of each tibia were measured using a dual-energy X-ray absorptiometer using a model for small subjects. Values are means and 95% CI ($n = 8$). *** Mean values were significantly different compared with the OVX + water group by Dunnett's test ($P < 0.001$). ††† Mean values were significantly different compared with the sham group ($P < 0.001$).

and Ca malabsorption; these phenomena might result from decreased levels of oestradiol in the blood⁽³⁰⁾. The present study found the mean values of Ca absorption and retention in OVX rats to be lower than in the sham group. However, the differences were not statistically significant. The reduction in Ca absorption and retention associated with OVX was restored by SAEAF treatment; this phenomenon might be explained by SAEAF having enhanced Ca absorption in the large intestine.

Previous research has shown that an increase in Ca absorption affected bone characteristics (e.g. bone Ca content and bone strength) in OVX rats⁽⁶⁾. The present study found that an improvement in Ca absorption because of SAEAF treatment in OVX rats caused an increase in tibial bone mineral content and bone mineral density. The SAEAF-treated groups also showed superior mechanical properties of the bone (maximal

load, energy to ultimate load, linear stiffness and the Young's modulus) compared with the OVX groups. In addition, microtomography scanning showed that OVX rats exhibited a lower trabecular bone volume, trabecular thickness and trabecular number, and higher trabecular separation in the distal femur, compared with the sham group. Treatment with SAEAF significantly reduced these signs of bone loss in OVX rats.

Previous studies have indicated that γ -GT is an osteoclastogenic factor. In addition, γ -GT is an ectopeptidase that catalyses the transfer of a γ -glutamyl moiety to an acceptor and acts in glutathione degradation and cysteine metabolism⁽³¹⁾. Research has demonstrated that both recombinant human γ -GT and purified γ -GT from the rat kidney promoted osteoclast formation through the expression of receptor activator for nuclear factor kappa-B ligand (RANKL) in bone marrow

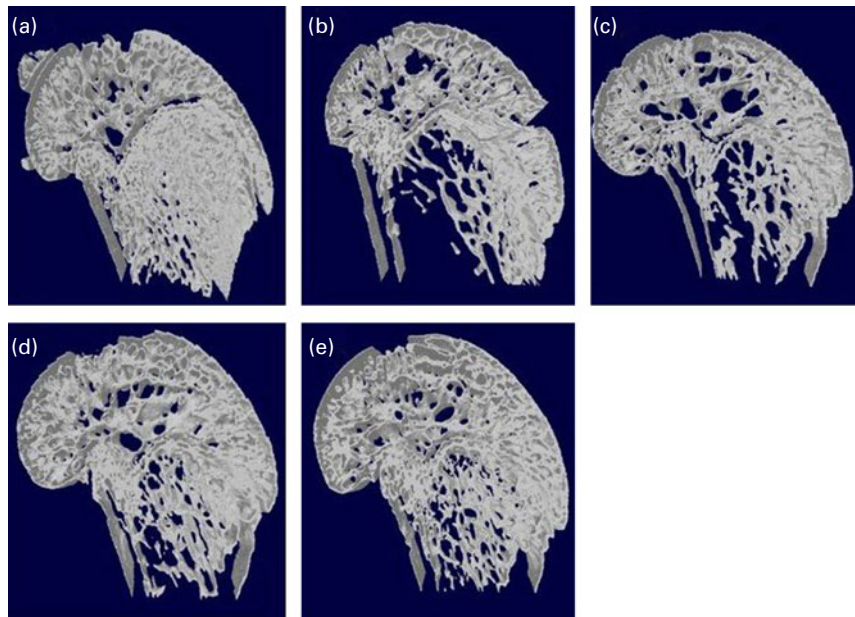


Fig. 6. Microtomography analysis of metaphysis of the distal femur in rats of different groups. (a) Sham + water group. (b) Ovariectomy (OVX) + water group. These rats showed a decrease in the trabecular bone volume. (c) OVX + standardised aqueous extract of *Anoectochilus formosanus* (SAEAF; 200 mg/kg) group. (d) OVX + SAEAF (400 mg/kg) group. (e) OVX + inulin (400 mg/kg) group. SAEAF (400 mg/kg) and inulin significantly prevented bone loss from OVX-induced osteopenia. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

Table 4. Effects of standardised aqueous extract of *Anoectochilus formosanus* (SAEAF) and inulin on the trabecular bone volume, number of trabeculae, thickness of the trabeculae and separation of trabeculae of the distal femoral metaphysis in ovariectomised (OVX) rats by microtomography analysis†

(Mean values with their pooled standard errors, n 8)

Items	Sham	OVX				Pooled SEM
		Water	SAEAF (200 mg/kg)	SAEAF (400 mg/kg)	Inulin (400 mg/kg)	
Trabecular bone volume (%)	45.4	30.2††	32.1	33.1*	33.5**	1.0
Number of trabeculae (no./mm)	4.8	3.2†††	3.5	3.6*	3.8*	0.1
Thickness of the trabeculae (µm)	98.2	93.4†††	94.9	97.5***	97.9***	0.5
Separation of trabeculae (µm)	168.7	408.1†††	364.6	354.3*	360.5*	10.7

Mean values were significantly different compared with the OVX + water group by Dunnett's test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Mean values were significantly different compared with the Sham group: †† $P < 0.01$, ††† $P < 0.001$.

‡ The trabecular bone microarchitecture of the distal femoral metaphysis was measured using a microtomography scanning, with an isotropic resolution of 18 µm in all three spatial dimensions.

stromal cells⁽³²⁾. Furthermore, Hiramatsu *et al.*⁽³³⁾ have demonstrated *in vivo* that transgenic mice that overexpressed γ -GT exhibited osteoporosis because of an increase in osteoclasts and bone resorption. Asaba *et al.*⁽³⁴⁾ have reported that urinary γ -GT levels were significantly associated with bone resorption, suggesting that γ -GT could be a useful diagnostic determinant for predicting osteoporosis.

Plasma OCN is a sensitive bone turnover marker⁽³⁵⁾. Urinary γ -GT is a potential marker of bone resorption⁽³⁴⁾. The present study found that both plasma OCN and urinary γ -GT levels were increased in OVX rats, indicating that OVX increased the bone turnover rate. Treatment with SAEAF significantly suppressed the increases in plasma OCN and urinary γ -GT levels in OVX rats. These results indicated that, in addition to enhancing Ca absorption, SAEAF inhibited bone turnover.

Administration of SAEAF significantly increased the caecal concentration of SCFA, especially of butyrate. Butyrate is recognised as a potential inhibitor of histone deacetylase activity⁽³⁶⁾. Rahman *et al.*⁽⁴⁾ have shown that butyrate inhibits osteoclastogenesis by modulating osteoclast-specific signals and histone deacetylase activity. In addition, histone deacetylase inhibitors such as butyrate have been reported to accelerate *in vitro* osteoblast maturation^(3,5). Treating OVX rats with SAEAF might have ameliorated osteoporosis, at least in part, by enhancing osteoblast maturation and inhibiting osteoclast differentiation through butyrate production.

Inulin, an IPAF, is the most well studied and established prebiotic⁽⁶⁾. Numerous rat studies have demonstrated that inulin-type fructants significantly increase Ca absorption and ameliorate the osteopenia that would otherwise be induced by OVX^(2,6). The present study found that inulin (400 mg/kg) and SAEAF (400 mg/kg) exerted approximately equivalent effects on Ca absorption and anti-osteoporotic activity. The SAEAF contained 0.25% IPAF; thus, the potency of IPAF was greater than that of inulin.

This was the first study to demonstrate that *A. formosanus* has a prebiotic character and promotes Ca absorption. Use of the herb as a folk remedy to treat stunted growth in children also suggests that it might enhance Ca absorption. The prebiotic action of *A. formosanus* has been shown to be significant in animals, but further research is required to confirm this

bioactivity in human subjects. Our preliminary *in vitro* study confirmed that the bifidogenic effect of SAEAF might be caused by polysaccharides. In future, research on *A. formosanus* would be regarding the chemical characters of the active ingredient, the bifidogenic mechanism and human trials.

In conclusion, the present results showed that SAEAF administration to OVX rats inhibited bone turnover, elevated intestinal Ca absorption and prevented Ca loss and deterioration of biomechanical strength. The present findings provide evidence that prebiotic activity might be involved in the anti-osteoporotic mechanisms of SAEAF; hence, SAEAF could be a potential candidate for the treatment of post-menopausal osteoporosis.

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